Flight Activity and Further Evidence for a Female-Produced Sex Pheromone of the Apple Leaf Midge, *Dasineura mali,* in Nova Scotia

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Abstract - The flight activity of the apple leaf midge, *Dasineura mali* (Kieffer), was determined in the field and laboratory. Males and females began emerging at the onset of the photophase. In the field, delta-shaped traps baited with 6 virgin females caught males exclusively, with the most abundant catches on the ground as compared to those at 1 and 2 m. Most males were captured at 1100 hours and males remained in the vegetation under the trees throughout the day. Sweep netting revealed that conspecifics mate in the vegetation under orchard trees in the morning hours, and females move from the ground to the orchard canopy to oviposit at 1000 hours. Gas chromatographic-electroantennographic detection (GC-EAD) analyses indicated the presence of an electrophysiologically active compound in solid phase microextraction (SPME) effluvial collections and pheromone gland extractions from virgin females.

Introduction

The apple leaf midge, *Dasineura mali* (Kieffer), is a relatively new insect in Nova Scotia (Rogers 1991) but has been established for some time in New Brunswick (MacPhee and Finnamore 1978). It has also been reported in Europe and New Zealand (Gagné 1989). *D. mali* is a specialist, leaf-feeding insect that attacks primarily young pubescent apple shoots. Larval feeding induces the leaves to curl tightly toward the midrib, forming distinctive reddish galls that desiccate after the larvae exit. Several authors have provided excellent illustrations and plates of leaf galls and sexual dimorphism as well as life history information (e.g., Barnes 1948, Berry and Walker 1989, Carl 1980, Helson 1972, Kolbe 1982, MacPhee and Finnamore 1978, Simova-Tosic 1974).

The gall midges are a huge family of insects with representatives from around the world. There are about 5000 described species, making cecidomyiids one of the largest dipteran families (Gagné 1994); however, this still probably represents "a small fraction of the number that

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must exist" (Gagné 1989). There is evidence of female-produced pheromones in twelve species (10 reviewed in Harris and Foster 1999). Tabuchi and Amano (2003) and Lenteren et al. (2002) have since illustrated the presence of pheromones in *Asteralobia sasakii* (Monzen) and the aphid predator *Aphidoletes aphidimyza* (Rondani), respectively. Of the species known to use sex attractant pheromones, only five pheromones have been chemically identified (Choi et al. 2004; Gries et al. 2000, 2002; Harris and Foster 1999; Hillbur et al. 1999).

The ultimate goal of work on this species is to identify the sex attractant pheromone produced by females that attracts males. This study is not the first to show evidence of a sex pheromone in *D. mali*. Work by Harris et al. (1996) and Heath et al. (1998) have also illustrated the attractiveness of virgin females to males. Furthermore, Harris et al. (1999) described the adult emergence and reproductive behavior of *D. mali* in New Zealand. The current study is unique in that it examines the diel emergence patterns over a continuous 72-hr period, describes the hourly location of males and females in the field, and examines the electrophysiological activity of glandular extracts.

Materials and Methods

Flight activity

Ten fully mature apple trees from a small commercial orchard (231 trees) in Berwick, NS, Canada (latitude, 45º02'45"N and longitude, 64º49'45"W) were randomly selected and sampled every hour from 0700 to 2000 hours for three days (25, 27, and 28 July 1995). The orchard contained three varieties: Red Delicious, McIntosh, and Cortland. Only the Red Delicious and McIntosh sections were sampled since *D. mali* appear to prefer these varieties over Cortland. Each hourly sample consisted of thirty 1.5-m strokes (parallel to the ground) with a 35-cm diameter sweep net within a 1.5-m radius of each of 5 tree trunks. Although this technique was used for both canopy and ground samples, each sample was taken from different trees to eliminate possible effects that sampling one area might have had on the other. Canopy sweeps were within the lower one-third of the canopy. The captured *D. mali* from each of the 5 ground and 5 canopy-sweep samples were counted and their sex was determined. The proportions presented in Figure 1 were calculated by dividing the "number of a specific sex captured over a particular time interval" by the total number of individuals captured for that day. These proportions were computed similarly for each day and the means calculated.

Insect rearing and hourly emergence patterns

Larvae collected from the same orchard on 19 June and 23 June 1996 were reared to adults in the laboratory. Galled leaves were collected,

thoroughly soaked with water, and placed in a sealed plastic container. This treatment encouraged mature larvae to exit the galls naturally, as they do after a rain storm in an orchard. Wandering larvae were rinsed from these leaves daily and placed in moist, sifted, 1:1 peat-sand mixture to allow pupation. To make handling and sexing of these fragile adults easier, pupae were sifted from the mixture a few days prior to emergence and isolated in 15-ml plastic screen-topped vials with ca. 3 cm of the mixture. The adults began emerging after 18 days in a ca. 80% relative humidity, LD 16:8 h photoperiod with the onset of the photophase at 0500 hours, and a 21 ± 1 ºC environment. Newly emerging individuals were counted and sexed every hour for 72 hours. The results were averaged over this consecutive three-day period. Insects used in electrophysiological studies were collected from the same location and treated in a similar manner.

Pheromone trapping

On 26 July 1997, six less-than-1-day-old, laboratory-reared, virgin, female adults were caged in delta traps (Cooper Mill Ltd., Madoc, ON, Canada) and placed at three different heights (ground, 1 m, and 2 m) along the tree trunk of ten randomly chosen apple trees in the above orchard (Sain and Kalode 1985). Traps were checked hourly from 0500 to 1300 hours and again at 2000 hours. Results were pooled and analyzed by one-way ANOVA followed by a Student-Newman-Keuls Test to separate means (SAS Institute Inc. 1999). The dependent variable was "number of males captured" and the independent variable was trap height. According to Levene's Test and the Brown and Forsythe's Test, variances were homogeneous (SAS Institute Inc. 1999) and the model errors were normally distributed; therefore, no transformations were made (Fry 1993).

Electrophysiological Recordings

The gas chromatograph coupled to an electroantennographic detector (GC-EAD) system was as previously described (Zhang and Polavarapu 2003, Zhang et al. 1997). A Hewlett Packard 5880 gas chromatograph equipped with a 30-m x 0.25-μm ID, 0.25-mm film-thickness, SE-30 capillary column (Alltech Associates, Inc.; initially at 150 ºC for 2 min, then programmed to 300 ºC at 10 ºC/min and held for 25 min) or a 30-m x 0.25-mm ID, 0.25-μm film-thickness, Stabilwax capillary column (Restek Corp.; initially at 120 ºC for 2 min, then programmed to 220 ºC at 10 ºC/min and held for 30 min) in the splitless mode, with nitrogen as the carrier gas, was used for GC-EAD analysis. The capillary column effluent and nitrogen makeup gas (10 ml/min) were split (≈ 1:1) by a Y GlasSeal capillary column connector (Supelco Inc., Bellefonte, PA) to a flame ionization detector (FID) and EAD. The body of a male *D. mali* was squeezed and then

positioned between two gold wire electrodes, which were immersed in saline-filled (0.9% NaCl) wells (1.25 mm ID; about 3 mm apart) in a small acrylic plastic holder (8 cm long x 0.8 cm wide x 0.6 cm thick). A small capillary tube (≈ 0.2 mm ID) was mounted in one well of the holder; it could slide back and forth so that the tips of antennae could make electrical contact with the saline solution in the capillary tube. The output recording electrodes were connected to a high-impedance 1:100 amplifier with automatic baseline drift compensation. The air-stream flowing over the antennae (about 500 ml/min) was humidified by bubbling it through distilled water before it entered the EAD interface. The antennal preparation was cooled to ≈ 5 ºC inside a condenser by circulating chilled water from a benchtop refrigeration unit (RTE-100, NESLAB instruments, Inc., Portsmouth, NH) through the insulation layer of the modified condenser (1.5 cm ID) containing the acrylic plastic holder mounted on top of the gas chromatograph (GC). An HP 3390 A integrator was used for EAD recording.

SPME sampling

One calling virgin female *D. mali* was introduced into a 1-ml glass vial with a Teflon-lined screwcap. A Polydimethyl siloxane-coated SPME fiber (film thickness 100 μm; Supelco Inc.) was conditioned in the GC injector (250 ºC) for 5 min and then passed through the small hole on the cap into the vial. The fiber was exposed for 24 hr to adsorb the volatiles, and the needle was then placed in the GC injection port for 2 min to desorb the analytes (see Zhang et al. 1999 for additional details of this technique).

Pheromone gland extraction

Pheromone gland extracts were obtained from five groups of newly emerged virgin females (10, 15, 15, 20, and 30 females per group) during the photophase. A female abdomen was compressed gently until the ovipositor everted from the abdominal tip. The ovipositor was then excised with small scissors into a conical glass vial containing ≈ 100 μl methylene chloride-methanol (3:1). The glands were soaked for at least 2 hr at room temperature. The solvent was removed and the glands were re-extracted with 100 μl methylene chloride-methanol. The combined solution was concentrated to ≈ 20 μl under a gentle stream of nitrogen gas and kept at -30 ºC in a freezer until analyzed.

Results

Flight activity

Flight activity, as indicated by the mean proportion of adults captured in sweep nets in a specific space, is presented in Figure 1. *Dasineura mali* females were most active in the canopy in the afternoon

with a slight tendency to move to the ground in the evening (Figs. 1a,b). Males and females were most often captured together on the ground in the mornings and occasionally at the end of the day (Fig. 1b). Male flight activity was greatest on the ground at 1000 hours.

Hourly emergence pattern

Males emerged slightly before females (Figs. 1 and 2). Male and female captures on the ground peaked in the morning with a smaller peak in the evening (Fig. 1b). Larvae that had been collected from the field in galled leaves and reared in the laboratory gave rise to adults in an approximate 1:1 sex ratio (Fig. 2 legend data).

Pheromone trapping

The majority of the males were captured in the ground traps between 0800 and 1300 hours with the peak at 1100 hours (Fig. 3). The 5-hour breadth of capture and emergence is similar to that determined in the laboratory, but the initiation time shifted from 0500 to 0800 hours.

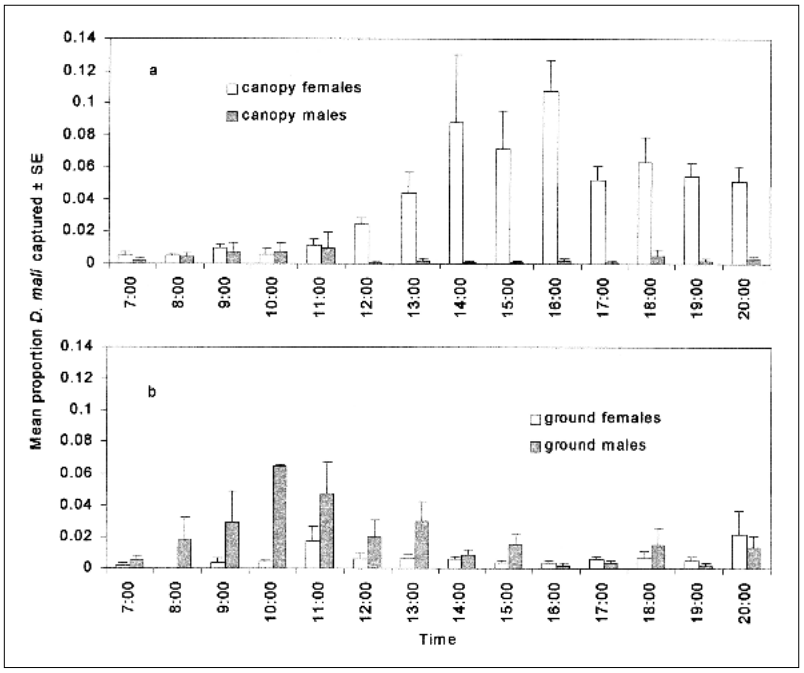

Figure 1. Mean proportion ± SE of *Dasineura mali* captured in the canopy of apple trees (a) and on the ground (b) for three days in July of 1995 in Nova Scotia, Canada. Samples were taken each hour between 0700 and 2000 hours for 3 days for each bar ($n = 3$). The total number of males and females captured on the 25th, 27th, and the 28th of July 1998 on the ground were 72 and 19, 80 and 26, and 50 and 19, respectively. The total number of males and females captured in the canopy on those same dates were 22 and 227, 12 and 100, and 4 and 140, respectively.

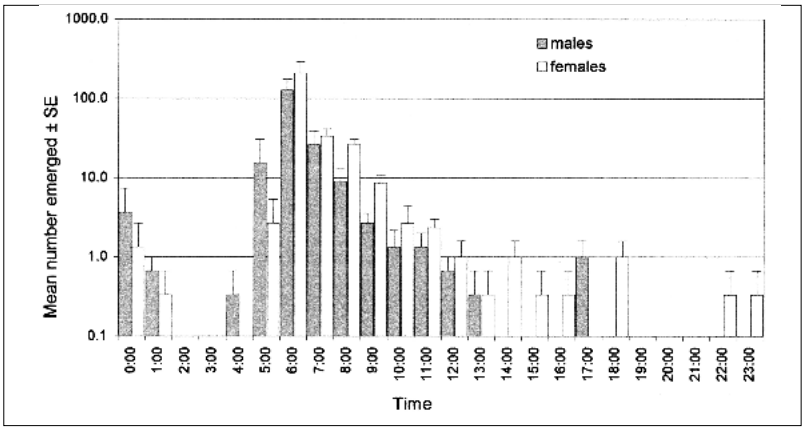

Figure 2. Semi-log plot of the mean number ± SE of *Dasineura mali* emerged with respect to time of day (checked every hour) for three consecutive days in the laboratory. The light phase was from 0500 to 2100 hours. Larvae were collected from an orchard in Nova Scotia, Canada in June 1996. The total number of males and females emerging for the first, second, and third consecutive days were: 212 males, 164 females; 263 males, 435 females; and 92 males, 264 females; respectively.

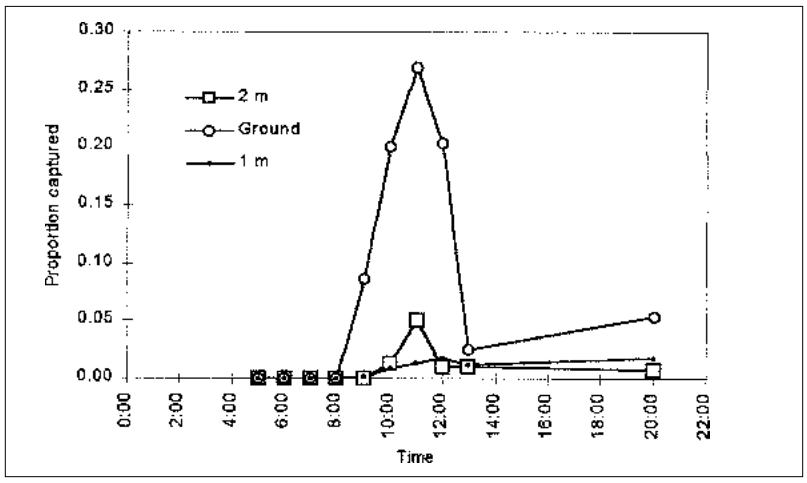

Figure 3. Mean proportion of adult *Dasineura mali* captured in delta traps positioned at 3 heights: ground level, 1 m, and 2 m along the trunks of apple trees in Nova Scotia, Canada in July of 1997. Traps were baited with 6 laboratory-reared virgin female *D. mali* and checked hourly between 0500 and 1300 and again at 2000 hours. The total number, mean number ± SE, and proportion of males captured in each treatment (summed over the time intervals) were respectively: ground: 1410, 141 ± 46 a, 0.84; 1 m: 121, 12 ± 3 b, 0.07; and 2 m: 156, 16 ± 9 b, 0.09; $n = 10$ traps per height; $P = 0.026$ according to ANOVA; means ± SE followed by the same letter are not significantly different according to Student-Newman-Keuls Test at $\alpha = 0.05$ (SNK test). Traps captured males exclusively.

Electrophysiological activity

The coupled GC-EAD analyses of female ovipositor extracts and SPME collections of effluvia demonstrated that male *D. mali* antennae consistently responded to a single compound (n = 5, representative example in Fig. 4). The amount of natural pheromone produced by female *D. mali* was below the FID detection threshold even though we injected a concentrated sample (about 100 female equivalents of the ovipositor extracts of mixed age). The EAD-active peak from female effluvial SPME collections and ovipositor extracts was observed at 14.18 min (C_{21} hydrocarbon, rt = 13.97 min) on a 30-m SE-30 capillary column and at 13.92 min (C_{22} hydrocarbon, rt = 13.85 min) on a 30-m Stabilwax column.

Discussion

In casual field observations throughout the season, *D. mali* females were often seen ovipositing on young leaf tips, but males were rarely observed in the canopy. On a number of occasions, females were observed calling while resting atop vegetation under orchard trees, a behavior common in female cecidomyiids (see photos in Harris and Foster 1999 and in Solinas and Isidoro 1991). In the vegetation under orchard trees, males were often observed flying upwind toward calling females, landing near them, performing a precopulatory behavior (digital video at http://

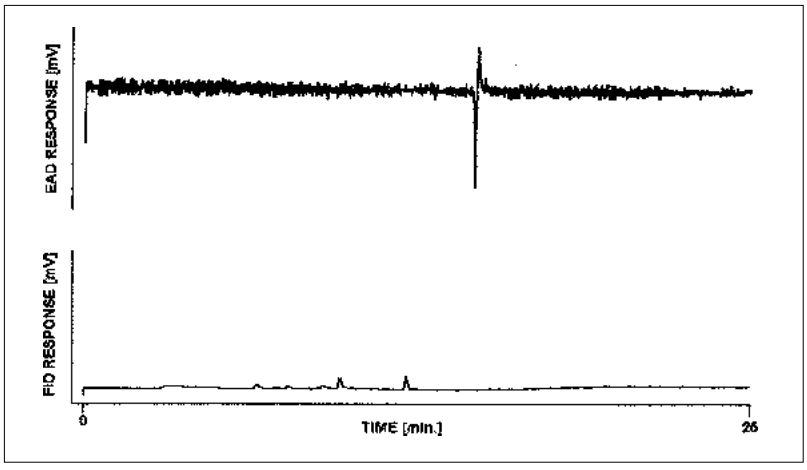

Figure 4. Representative (n = 5 different male antennae), simultaneous responses of the gas chromatograph's (GC) flame ionization detector and the electroantennographic detector using antennae of male apple leaf midge, *Dasineura mali*, to volatile effluvium collected by solid phase microextraction (SPME) from one virgin female. The GC was a Hewlett Packard 5880 gas chromatograph equipped with a 30-m x 0.25-mm ID, 0.25-mm film-thickness, SE-30 capillary column programmed initially at 150 ºC for 2 min, then programmed to 300 ºC at 10 ºC/min and held for 25 min.

www.personal.psu.edu/jjh308/alm.avi), and eventually mating. The general observation that mating occurs on the ground was verified in our sweep-net study (Fig. 1) and in the capture of the majority of males in ground traps (Fig. 3). Harris et al. (1999) found a similar result from traps baited with 10 virgin females of *D. mali* in New Zealand. In their study, about 90% of the males were captured in traps placed between 10 and 80 cm above the ground. Furthermore, work by Harris et al. (1999) and Galanihe and Harris (1997) might provide a proximate explanation for the tendency of females to move to the trees after mating. In those studies they found that female *D. mali* were strongly attracted to apple volatiles, and the intensity of the attraction increased with time of day.

The adults of *D. mali* only live approximately 3–4 days (Barnes 1948, Helson 1972); therefore, it is necessary for them to mate quickly and oviposit their eggs. Under our laboratory conditions, observations have shown that *D. mali* tend to mate less than one hour after eclosion (unpublished data). This was also the case for *D. mali* in New Zealand (Harris et al. 1999). Their ability to mate quickly is due to their ability to synchronize their emergence both temporally (Figs. 1 and 2) and spatially (Fig. 1). The tendency for females to remain on the ground until they are mated keeps virgin adults close in space and decreases the time required to find a mate. Males emerging even minutes ahead of other males will increase their chances of finding a receptive female. This force could select for earlier emerging males and thus the occurrence of a temporally isolated population as suggested by Figure 2. It is important to note that female proportions (Fig. 1b) on the ground were possibly under-represented because females tend to fall down from their perches when disturbed by the sweep net, whereas males fly upwards when disturbed and are more likely to become entrapped in the net.

Harris et al. (1999) also observed the emergence timing of *D. mali* in New Zealand and found that no adults eclosed after 1200 hrs. However, we found that some adults of the Nova Scotia population do indeed emerge during the scotophase (Fig. 2).

The amount of natural pheromone produced by female *D. mali* is below the FID detection threshold; therefore, any attempt to identify the pheromone with conventional techniques, like mass spectrometry and nuclear magnetic resonance, may require at least a thousand virgin females. However, if one trusts the comparison of EAD-active peak retention times with authentic standards of other known cecidomyiid pheromone components on several polar and non-polar capillary columns, the work may require fewer virgin females provided that the compound is similar to those already identified. Microchemical reactions followed by behavioral and electrophysiological testing may also be helpful in determining the functional groups present without the need for a lot of material. Furthermore, characterization of other more abun-

dant, but biologically inactive, compounds in the gland extracts may provide important clues to the structure of biologically active, but depleted compounds.

Virgin females may serve as an effective lure for monitoring the emergence of the overwintering generation (Sain and Kalode 1985), at least until the sex pheromone can be identified. Clearly, if this technique is used, the traps should be placed on the ground where males are most abundant.

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